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Engagement of ICAM-3 Provides a Costimulatory Signal for Human Immunodeficiency Virus Type 1 Replication in both Activated and Quiescent CD4$^+$ T Lymphocytes: Implications for Virus Pathogenesis

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Human immunodeficiency virus type 1 (HIV-1) replication is regulated by several extracellular signals. We demonstrate that intercellular adhesion molecule 3 (ICAM-3) acts as a costimulating molecule to increase HIV-1 transcription and viral production, a process allowing productive infection of quiescent CD4$^+$ T lymphocytes. The present work suggests an important role for ICAM-3 in HIV-1 replication.

Human immunodeficiency virus type 1 (HIV-1) replication is intimately linked to the cellular activation state and is controlled at the transcriptional level by numerous cell factors that bind to the regulatory region located in the long terminal repeat (LTR). Because a number of *cis*-acting motifs located within the HIV-1 LTR are also found in the regulatory region of genes induced after T-cell activation, extracellular signals that mediate T-cell activation and lymphokine gene expression also regulate HIV-1 gene expression. It has been shown that antibody-mediated signaling through the T-cell receptor (TCR)/CD3 complex activates HIV-1 transcription, and coengagement of the costimulatory molecule CD28 further augments virus gene expression (19, 47). An increasing number of accessory cell surface molecules are also involved in upregulation of T-cell activation (reviewed in reference 51). For example, we have recently demonstrated that CD43 functions as a potent costimulatory molecule for TCR/CD3-dependent induction of HIV-1 LTR-driven transcription and virus production (3). Another molecule of great interest in that respect is the intercellular adhesion molecule 3 (ICAM-3, or CD50), a 120-kDa, highly expressed cell surface antigen restricted to the leukocyte cell lineage (1, 20, 24). ICAM-3 exerts numerous functions in T-cell adhesion, polarization, and activation during the normal immune response (6, 10, 18). This molecule possesses a costimulatory potential for both resting and activated T lymphocytes (5, 22, 29). Contrary to many other costimulatory molecules, ICAM-3 is expressed constitutively at a very high level on resting T lymphocytes and consequently could play an essential role in the initiation of the immune response. Previous studies suggest that ICAM-3 might affect HIV-1 biology. For example, ICAM-3 has been proposed to play a role in HIV-1 entry, since some antibodies specific for ICAM-3 significantly inhibit the early events in the virus life cycle (44). Moreover, ICAM-3, by acting as a natural counterreceptor for DC-SIGN, has been thought to be involved in HIV-1 transmission by dendritic cells through its strong interaction with the viral envelope protein gp120 (25, 40). However, recent studies indicate that DC-SIGN/ICAM-3 interactions do not promote DC-SIGN-mediated virus transmission (26, 52). To shed light on other possible modulatory effects of ICAM-3 on HIV-1 biology, we investigated whether the ICAM-3-mediated signal transduction pathway can affect HIV-1 transcription and replication.

ICAM-3 engagement enhances HIV-1 transcriptional activity induced by TCR/CD3 ligation. We first analyzed the effect of antibody-mediated ICAM-3 cross-linking on HIV-1 LTR-driven reporter gene activity. Freshly isolated purified CD4$^+$ T cells were transfected by the Nucleofector technology (23) with an HIV-1 LTR-oriented luciferase vector (pLTRX-LUC) (43), either used alone or in combination with a Tat expression vector (pCEP4-Tat) (14), before treatment with various combinations of anti-TCR/CD3 (OKT3), anti-ICAM-3 (ICR-1 or ICR-6.2) (42, 50), and anti-CD28 (9.3) antibodies. The anti-CD28 antibody was used as a positive control throughout the following experiments. A modest, but nevertheless statistically significant, HIV-1 LTR-driven transcriptional activation was observed following TCR/CD3 and ICAM-3 or CD28 coengagement (Fig. 1A). The low costimulatory capacity of ICAM-3 is probably linked with the very weak transcriptional activity of the pLTRX-LUC molecular construct in primary CD4$^+$ T cells. However, in the presence of the viral transactivating protein Tat, which induced a 100-fold increase in reporter gene activity, the engagement of ICAM-3 or CD28 by specific antibodies along with TCR/CD3 stimulation induced a stronger increase in HIV-1 LTR-driven activity (Fig. 1A). In both cases, treatment with anti-ICAM-3 or anti-CD28 antibodies alone did not induce any transcriptional activation (data not shown for anti-CD28). A detectable ICAM-3-dependent increase in HIV-1 LTR-oriented reporter gene activity was seen even with an OKT3 concentration that is not sufficient to mediate any activation by itself (i.e., 0.1 μg/ml), suggesting that ICAM-3 engagement could lower the TCR/CD3 signaling threshold. In addition, antibody-mediated ligation of TCR/CD3 and ICAM-

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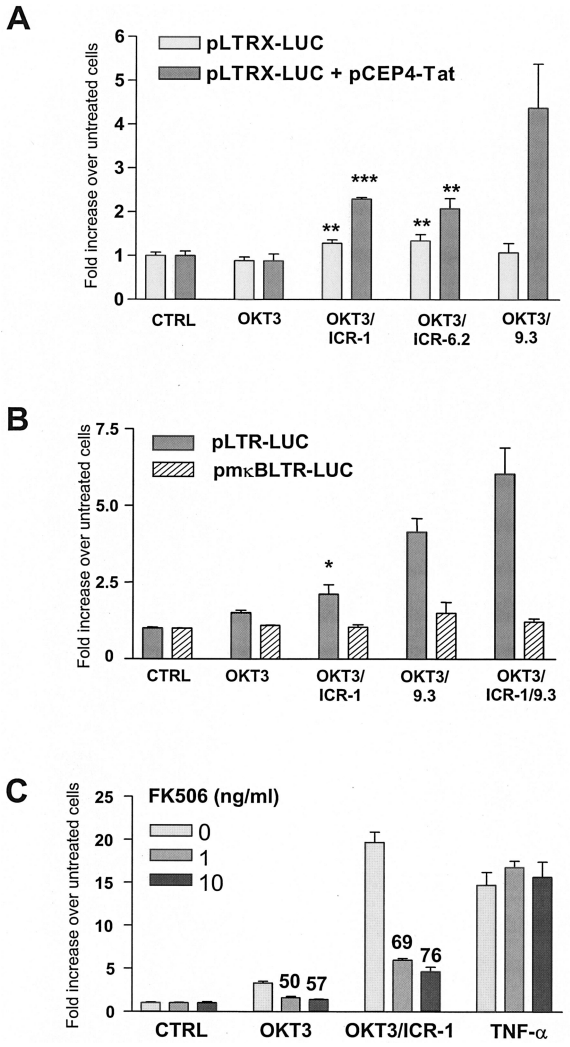

FIG. 1. ICAM-3 provides a cosignal that enhances TCR/CD3-mediated induction of HIV-1 LTR activity, a signal mediated through NF-κB- and calcineurin-dependent pathways. (A) Freshly isolated $CD4^+$ T cells were transiently transfected with pLTRX-LUC (3 μg) or cotransfected with pLTRX-LUC and a Tat expression vector (pCEP4-Tat) (1 μg). Next, cells were either left untreated (CTRL) or treated for 15 h with OKT3 (0.1 μg/ml), used either alone or in combination with anti-ICAM-3 (ICR-1 or ICR-6.2) or anti-CD28 (9.3) antibodies (1 μg/ml) before monitoring luciferase activity. (B) Jurkat cells were transiently transfected with wild-type or NF-κB mutated HIV-1 LTR-driven luciferase vectors before stimulation for 8 h with the indicated antibodies. (C) Jurkat cells were transiently transfected with the pκBTATA-LUC molecular construct and were next pretreated for 60 min with FK506 (1 or 10 ng/ml) before treatment for 8 h with OKT3 (0.5 μg/ml) used either alone or combined with ICR-1 (1 μg/ml) or tumor necrosis factor alpha (TNF-α) (10 ng/ml). The percentage of inhibition by FK506 is indicated at the top of the appropriate bars. Results are presented as *n*-fold induction in luciferase activity over untreated samples. Data shown are from triplicate samples and are representative of three independent experiments. The statistical significance of differences between anti-CD3 and anti-CD3/anti-ICAM-3 treatments is indicated at the top of the bars: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t* test).

3 did not lead to induction of a reporter gene placed under the influence of a cyclic AMP-responsive element (pCRE-LUC) (data not shown), therefore indicating that the observed phenomenon is not simply a consequence of T-cell activation.

NF-κB and NFAT are involved in ICAM-3 costimulating effect on HIV-1 LTR activity. The regulation of HIV-1 transcription that is seen with several stimuli, including TCR/CD3 and CD28 ligation, involves the NF-κB complex and nuclear factor of activated T cells (NFAT) family members, which bind to the HIV-1 enhancer region (21, 31, 37). To assess the involvement of the NF-κB transcription factor in the observed costimulating activity of ICAM-3, we used an HIV-1 LTR-based reporter construct bearing mutated NF-κB binding sites (i.e., pmκBLTR-LUC) (28). The increase in HIV-1 LTR activity mediated by coligation of CD3 and ICAM-3 or CD28 was almost totally abolished in Jurkat cells transfected with this molecular construct (Fig. 1B). It should be noted that an additive effect was obtained when using saturating concentrations of anti-ICAM-3 and anti-CD28 antibodies in combination with TCR/CD3 engagement (Fig. 1B), suggesting that the ICAM-3 costimulating effect is independent from CD28 and most likely acts through distinct signaling pathways. In order to assess the involvement of NFAT, the effect of the calcium-calcineurin inhibitor FK506 was then analyzed using pκB-TATA-LUC, a molecular construct containing the minimal HIV-1 enhancer region and a TATA box (46). This inhibitor caused a 76% decrease in the transcriptional activity resulting from TCR/CD3 and ICAM-3 coengagement (Fig. 1C), therefore suggesting the involvement of a calcineurin-dependent signal transducer such as NFAT. The FK506-induced inhibition observed following TCR/CD3 and ICAM-3 costimulation was more severe than with the engagement of TCR/CD3 alone (76 versus 57%), suggesting that both TCR/CD3- and ICAM-3-mediated signals follow calcineurin-dependent pathways. The tumor necrosis factor alpha-induced activation, which is known to involve NF-κB but not NFAT, was unchanged by FK506, confirming the specificity of this inhibitor.

To further document the implication of NF-κB and NFAT transcription factors in the HIV-1 transcriptional activation induced by TCR/CD3 and ICAM-3 coengagement, we were next interested in assessing whether the ICAM-3-mediated signaling pathway could modulate the level of HIV-1 enhancer-bound protein complexes. To this end, mobility shift assays were conducted with a labeled probe containing the complete enhancer region of the HIV-1 LTR (−107 to −77) (3). Incubation of this probe with extracts from anti-TCR/CD3-treated $CD4^+$ T cells led to the formation of a specific broad signal (Fig. 2A). It has already been reported that this signal can be the result of overlapping NF-κB and NFAT complexes (3, 4). The binding of NF-κB was confirmed by supershift with anti-NF-κB p50 antibodies (Fig. 2A). As for NFAT, it has been shown to bind as a dimer to the enhancer κB sites (27), but its binding can be difficult to visualize by electrophoretic mobility shift assay (EMSA) in the presence of high amounts of NF-κB. Consequently, we instead monitored the nuclear translocation of NFAT by using a NFAT-specific probe (Fig. 2B). Supershift assays indicate the preferential activation of NFAT1. Thus, EMSA and supershift assays performed with both probes indicate that both NF-κB and NFAT binding are increased by coengagement of TCR/CD3 and ICAM-3 when compared to the ligation of TCR/CD3 complex alone. The AP-1 transcription factor has also been shown to play a role in HIV-1 transcriptional regulation through binding sites located both in the modulatory region and the untranslated leader sequence (11,

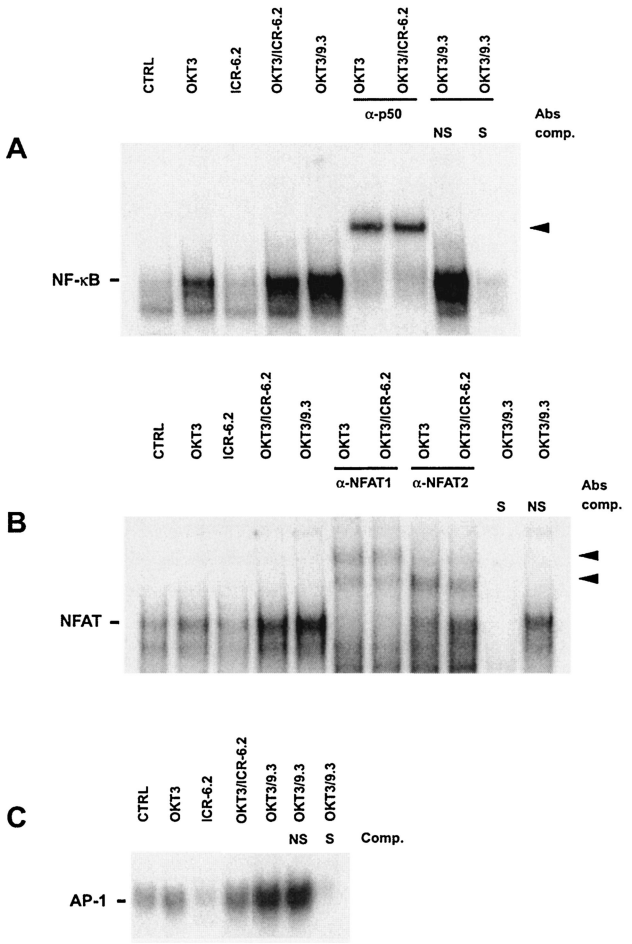

FIG. 2. Coligation of ICAM-3 and TCR/CD3 complex results in nuclear translocation of NF-κB, NFAT, and AP-1. Human $CD4^+$ T cells were incubated for 7 h with the indicated antibodies before preparing nuclear extracts. EMSAs were then carried out using an HIV-1 enhancer probe (A), a NFAT-specific probe (B), or an AP-1-specific probe (C). Competitions were performed with a 100-fold excess of either specific (S) or nonspecific (NS) oligonucleotides. Supershift assays were performed with anti-NF-κB p50, anti-NFAT1, or anti-NFAT2 antibodies (Abs). NF-κB-, NFAT-, and AP-1-specific complexes are indicated. Arrows on the right side indicate supershifted bands. Controls (CTRL) consisted of cells treated with an isotype-matched irrelevant antibody (i.e., immunoglobulin G2a).

41, 53). By using an AP-1-specific probe, we demonstrated that the AP-1 binding activity augments upon coligation of TCR/CD3 and ICAM-3 (Fig. 2C). A physical association between the two AP-1 components, *c-Fos* and *c-Jun*, and NF-κB was reported, leading to a synergistic activation of the HIV-1 LTR through NF-κB sites (53). Whether AP-1 is involved in HIV-1 LTR costimulation by ICAM-3 through its own binding sites or though a complex formation with NF-κB remains to be determined.

HIV-1 gene expression and virus production are both increased following occupancy of ICAM-3 and TCR/CD3 in primary human cells. To assess the ICAM-3-dependent enhancement of HIV-1 transcriptional activity in the context of an integrated viral genome, we used recombinant luciferase-encoding HIV-1 particles that were pseudotyped with the vesicular stomatitis virus G (VSV-G) envelope protein (3). When primary $CD4^+$ T-cell blasts were infected with such viruses, a threefold increase in HIV-1 transcriptional activity was observed following treatment with OKT3 and the anti-ICAM-3 antibody ICR-6.2 (Fig. 3A). Next, we wanted to see if the observed upregulatory effect on HIV-1 transcription could translate to an enhancement of virus production. To this end, mitogen-activated human peripheral blood mononuclear cells (PBMCs) were inoculated with replication-competent virions (i.e., HIV-1_{NL4-3}) (2) and then treated with combinations of plate-bound antibodies specific for TCR/CD3, ICAM-3, or CD28, and virus production was monitored at various times postinfection. The HIV-1 production seen in cells treated with anti-TCR/CD3 and anti-ICAM-3 antibodies was two- to threefold higher than that in cells treated with OKT3 alone (Fig. 3B).

Coligation of ICAM-3 and TCR/CD3 facilitates productive infection of resting $CD4^+$ T cells. It is now well documented that infection of quiescent $CD4^+$ T cells is not productive due to blocks in the viral life cycle at steps prior to the integration of the viral genome into the host cell chromosome (54, 55). Since ICAM-3 is constitutively expressed at high levels on resting T cells, ICAM-3 signaling could play a role in overcoming this blockade, thus allowing HIV-1 transcription in a newly infected quiescent cell. This possibility was tested by infecting freshly isolated, unstimulated PBMCs or purified $CD4^+$ T cells. A small but detectable virus production was observed even in untreated control cells (Fig. 3C), which can be explained by the presence of a few activated cells in the PBMC population (which contains T and B lymphocytes but also antigen-presenting cells). However, virus production was much more important in resting PBMCs upon coengagement of TCR/CD3 and ICAM-3 and was similar to coligation of TCR/CD3 and CD28 (Fig. 3C). Furthermore, when using purified quiescent $CD4^+$ T cells, no measurable HIV-1 production could be detected in either untreated cells or in cells treated with OKT3 alone, thus confirming the quiescent state of the cells. In contrast, a very robust virus production was observed in conditions where both TCR/CD3 and ICAM-3 were engaged, resulting (9 days postinfection) in a production of viral p24 that was 200-fold higher than that in cells subjected to OKT3 treatment alone (Fig. 3D). These results suggest that ICAM-3 engagement facilitates the productive infection of quiescent $CD4^+$ T lymphocytes.

In this report, we show for the first time that ICAM-3 can act as a costimulatory molecule to enhance HIV-1 transcriptional activity in primary $CD4^+$ T cells and can lower the threshold of signaling through the TCR/CD3 complex necessary to achieve activation of viral replication. Recent studies indicate that HIV-1 replication in quiescent cells is impaired by a significant decay of the genome during reverse transcription, a very slow process in such cells (39). Treatment with anti-CD3 antibodies alone was not sufficient to induce a productive virus infection. Indeed, stimulation of the TCR/CD3 complex alone engages T lymphocytes into the G_1a phase of the cell cycle, whereas progression to the G_1b phase is required for completion of the reverse transcription process (32). Costimulation through ICAM-3 was very effective in overcoming this block, leading to highly productive HIV-1 infection. The ICAM-3-mediated permissiveness of resting $CD4^+$ T cells to productive infection by HIV-1 is most likely due to the described effects of ICAM-

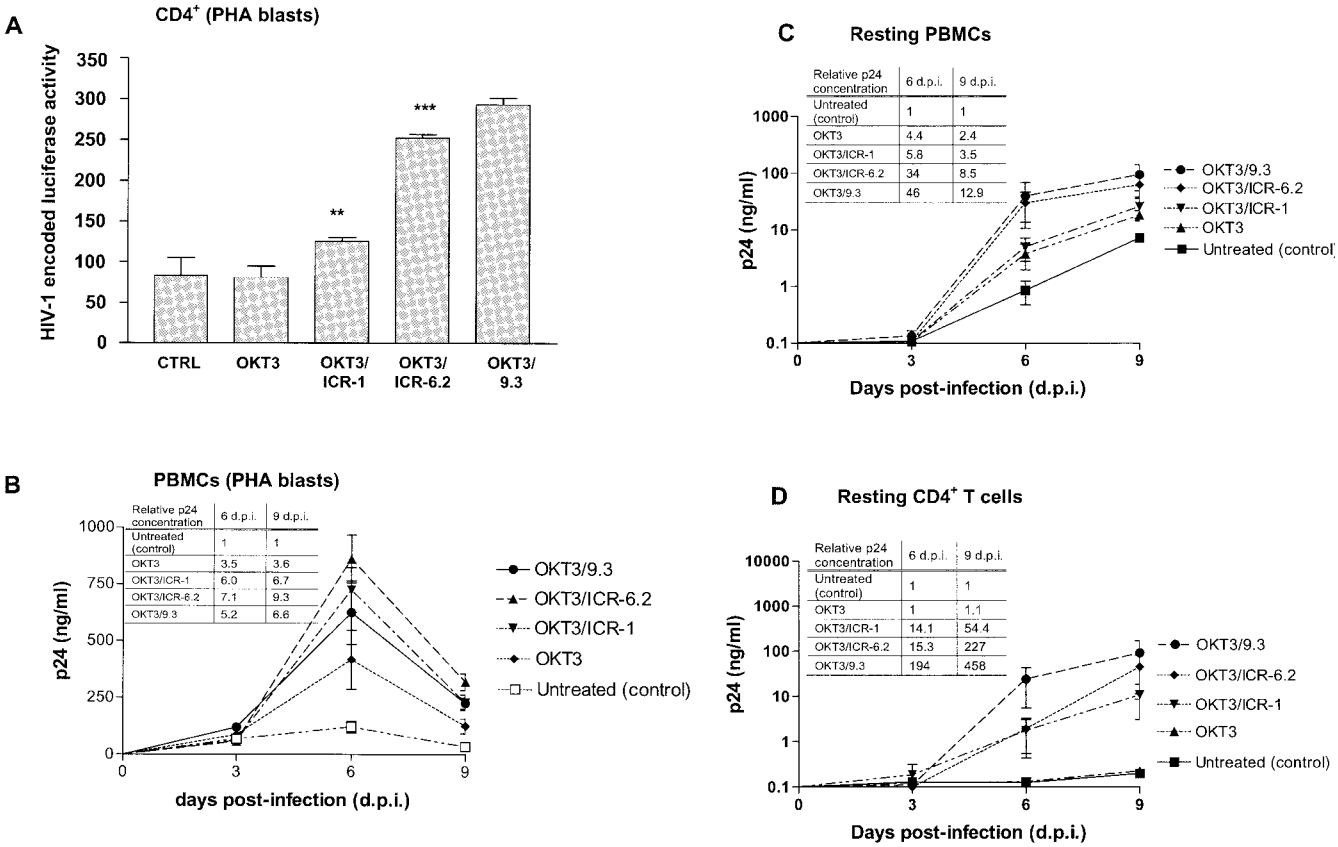

FIG. 3. Coligation of ICAM-3 and TCR/CD3 leads to higher HIV-1 gene expression and virus production as well as to productive infection of quiescent primary human cells. (A) $CD4^+$ T cells previously activated for 48 h with phytohemagglutinin (PHA) (1 μg/ml) were infected with luciferase-encoding HIV-1 particles pseudotyped with the VSV-G envelope protein. Forty-eight hours postinfection, cells were stimulated for 24 h with the indicated antibodies before assessing virus-encoded reporter gene activity. The statistical significance of differences between OKT3 and OKT3/anti-ICAM-3 treatments is indicated at the top of the appropriate bars: **, $P < 0.01$; ***, $P < 0.001$ (Student t test). Activated PBMCs (PHA blasts) (B), freshly isolated PBMCs (C), or freshly isolated $CD4^+$ T cells (D) were initially infected with fully competent $HIV\text{-}1_{NL4\text{-}3}$ particles and were next stimulated 4 h postinfection with the indicated plate-bound antibodies. Virus production was monitored at days 3, 6, and 9 postinfection by measuring cell-free p24 antigen (8). The n-fold increase in p24 concentration over untreated cells (arbitrarily considered as 1) is presented in an insert. Data shown are from triplicate samples and are representative of three independent experiments.

3/CD3 engagement on cell size and surface expression of activation markers CD25 and CD69 (5), since these effects suggest a progression of cells to the G_1b phase. Another explanation may reside in a possible ICAM-3-dependent induction of NFAT, since the forced expression of NFAT was shown to overcome reverse transcription blockade and induce a highly permissive state for HIV-1 replication in primary $CD4^+$ T cells (30). Constitutively expressed on the surfaces of resting T cells, ICAM-3 plays a crucial role in the activation of these T cells by antigen-presenting cells expressing LFA-1. Actually, ICAM-3 expression is acquired by early T-cell progenitors ($CD34^+$) and maintained during thymic development (35). Hence, ICAM-3 could play a major role in the propagation of HIV-1 by allowing infection of resting circulating $CD4^+$ T cells but also of thymocytes and even the earliest T-cell progenitors, thus contributing to thymus dysfunction and involution observed in HIV-1-infected patients (7, 45).

The observed ICAM-3 costimulating potential in quiescent $CD4^+$ T cells also suggests a possible influence on the reactivation of latently infected resting $CD4^+$ T cells. The latent HIV-1 reservoir in quiescent $CD4^+$ T cells is thought to represent a major obstacle for the clearance of this retroviral infection by current highly active antiretroviral therapy (9, 15, 38, 56). Latent virus can be reactivated by environmental stimuli that trigger T-cell transcription (16, 17, 36, 48). Recently, immense efforts have been deployed to design immune-based activation therapies in order to accelerate virus clearance and hopefully eradicate HIV-1 infection (33, 34). Combinations of anti-ICAM-3 and anti-TCR/CD3 antibodies could be included in such therapeutic strategies.

In conclusion, we established that ICAM-3 may be a biologically significant molecule playing a role in HIV-1 transcription and replication, particularly in resting $CD4^+$ T cells. Costimulatory molecules may become crucial when the TCR/CD3 signaling pathway is impaired by interaction of the viral gp120 molecule with CD4 (12, 13). Since we demonstrated that ICAM-3 costimulation needs only a minimal TCR/CD3 engagement, this molecule could modulate HIV-1 gene expression either in cooperation with CD28 or in CD28 null cells, which are predominantly found in the aging immune system (49).

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